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A change in soybean agglutinin binding patterns of bovine milk fat globule membrane glycoproteins during early lactation

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Milk fat globule membrane (MFGM) glycoproteins were prepared from bovine milk at different stages of early lactation. Western blot analyses using several lectins revealed that reactivity of MFGM glycoproteins, especially 47K and 80K bands, to soybean agglutinin (SBA) remarkably increased during the lactation, while no change was observed for *Ricinus communis* agglutinin-I (RCA-I) binding. Sialidase treatment of MFGM glycoproteins revealed that the number of SBA-positive bands and the amount of SBA-positive oligosaccharides in these bands are increased during the lactation. Since SBA binds *N*-acetylgalactosamine terminated oligosaccharides, the results indicated that *N*-acetylgalactosaminylation of bovine MFGM glycoproteins is stimulated during the lactation.

N-Linked sugar chains; *N*-Acetylgalactosaminylation; Sialylation; Lactation

1. INTRODUCTION

The mammary gland undergoes profound morphological and biochemical changes for milk production during pregnancy of the mammalian female. Following lactation, the gland synthesizes enormous amounts of soluble glycoproteins such as α-lactalbumin, lactotransferrin and caseins, and of a number of glycoproteins which are constituents of the MFGM [1–3]. It has been shown that the synthesis of some of the milk proteins is regulated by lactogenic hormones and growth factors [4,5]. Therefore, this tissue has attracted some biochemists to study the hormonal regulation of protein glycosylation. Several studies showed that activities of some of the key glycosyltransferases involved in the synthesis of dolichol-linked oligosaccharides, which are the precursors of *N*-linked oligosaccharides, and of glucosidase I, which is involved in the processing of *N*-linked oligosaccharides, are increased during the gland development [6,7]. These results suggest that protein *N*-glycosylation in the mammary gland is stimulated during lactation.

In the present study, we compared the glycosylation patterns of bovine MFGM glycoproteins during lactation by lectin blot analyses. The MFGM is derived from the apical plasma membranes of differentiated mammary epithelial cells during the secretion of milk triacylglycerols [8]. Glycoproteins included in each MFGM preparation, which was obtained from bovine milk at different stages of lactation, were subjected to Western blot analyses using several lectins. The results show that the SBA binding patterns of MFGM glycoproteins were dramatically changed during early lactation.

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Abbreviations: CBB, Coomassie Brilliant Blue; MFGM, milk fat globule membrane; PVDF, polyvinylidene difluoride; RCA-I, *Ricinus communis* agglutinin-I; SBA, soy-bean agglutinin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin.

2. MATERIALS AND METHODS

2.1. *Purification of bovine MFGM glycoproteins*

Fresh bovine milk was obtained from a Holstein cow at days 0, 1, 3 and 5 after parturition. MFGM was prepared by the method described previously [9]. In brief, fresh bovine milk was centrifuged at 4,000 × *g* for 15 min at 4°C, and the floating cream layer was washed several times with water to remove adhesive skim milk components. Washed cream was diluted with one volume of 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl followed by freezing and thawing several times. The aqueous butter milk was centrifuged at 100,000 × *g* for 60 min, and the membrane layer was obtained in the interphase of the solution. The membrane fraction was dialyzed against water, and the MFGM glycoproteins were solubilized in 10 mM Tris-HCl buffer, pH 8.3, containing 1% SDS and 2% β-mercaptoethanol (TSM) by incubating at 80°C for 10 min.

2.2. *SDS-PAGE and lectin blotting*

SDS-PAGE was performed according to the method of Laemmli [10] by applying 2 μl of the MFGM glycoprotein solution; proteins were then transferred to PVDF filters [11]. Western blot analysis using wheat germ agglutinin (WGA), RCA-I or SBA was performed as described by Hawkes [12]. After blocking with 1% bovine serum albumin, filters were incubated successively with lectin, rabbit anti-lectin antiserum and peroxidase-conjugated anti-rabbit IgG antibody. Finally, filters were incubated with chromogenic peroxidase substrate

4-chloro-1-naphthol to detect lectin-bound glycoproteins. In some experiments, filters were treated with 0.5 units of *Arthrobacter ureafaciens* sialidase in 100 μl of 0.5 M acetate buffer, pH 5.0, prior to incubation with SBA, and the glycoprotein samples (200 μg) were treated with (i) 1.5 units of *N*-glycanase in 30 μl of 0.5 M Tris-HCl buffer, pH 8.3; (ii) 1.0 unit of jack bean β-*N*-acetylhexosaminidase in 50 μl of 0.3 M citrate-phosphate buffer, pH 4.0, containing 100 μg of γ-galactonolactone; (iii) 0.05 unit of diplococcal β-galactosidase in 50 μl of 0.3 M citrate-phosphate buffer, pH 6.0; (iv) 1.0 unit of coffee bean α-galactosidase in 50 μl of 0.3 M citrate-phosphate buffer, pH 6.5, containing 100 μg of γ-galactonolactone, or (v) 0.1 unit of *Acremonium* sp. α-*N*-acetylgalactosaminidase in 50 μl of 0.3 M citrate-phosphate buffer, pH 4.5, at 37°C for 24 h prior to the analysis.

2.3. *Liberation of* N-*linked sugar chains from glycoproteins*

MFGM Glycoproteins (2 mg), which were dried thoroughly over P_2O_5, were subjected to hydrazinolysis for 15 h as described previously [13]. After *N*-acetylation, the released oligosaccharides were reduced with NaB^3H_4 to obtain tritium-labeled oligosaccharides. The radioactive oligosaccharides were subjected to high-voltage paper electrophoresis in pyridine-acetate buffer 3:1:387 (pyridine/acetic acid/water, by volume), pH 5.4, at 70 V/cm for 90 min as described previously [14]. Radioactive mono- and disialyl biantennary complex-type sugar chains were prepared from human transferrin by hydrazinolysis [15] followed by reduction with NaB^3H_4.

2.4. *SBA-agarose column chromatography and affinity precipitation*

Radioactive oligosaccharides on an SBA-agarose column were eluted with phosphate-buffered saline, pH 7.4, followed by the same buffer containing 100 mM *N*-acetylgalactosamine. MFGM glycoproteins were dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 and incubated with SBA-agarose beads at 37°C for 6 h with gentle shaking. After centrifugation at 3,000 × *g* for 15 min, glycoproteins bound to SBA-agarose beads were precipitated. The bound glycoproteins were extracted with TSM by incubating at 80°C for 10 min and subjected to SDS-PAGE followed by Western blot analysis using a rabbit anti-bovine CD36 antibody.

3. RESULTS AND DISCUSSION

Protein concentrations of MFGM preparations obtained from 100 ml each of day 0, day 1, day 3 and day 5 milk samples were 13.8, 8.3, 6.6 and 6.5 mg/ml (total 1 ml), respectively, as determined by the method of Lowry et al. [16]. When 2 μl of each preparation was subjected to SDS-PAGE followed by staining with Coomassie brilliant blue (CBB), it was shown that each preparation contains 47K, 50K, 66K, 80K and 120K bands, which are indicated by a, b, c, d and e in each panel in Fig. 1, as common components and a few different minor bands with molecular sizes under 40K (Fig. 1A). However, day 0- and day 1-derived samples contained additional 28K and 56K bands, which are indicated by arrowheads in Fig. 1A, identified as the light and heavy chains of immunoglobulins (data not shown). Because day 0-milk contains a large amount of immunoglobulins, they are often co-purified with MFGM proteins [17]. The 160K band as indicated by f was present in day 0- and day 1-derived preparations but less or none in day 3- and day 5-derived preparations (Fig. 1A). The band f was stained relatively strongly with CBB but did not react with lectins. Therefore, if the immunoglobulin and minor component bands were excluded, it was considered that each MFGM preparation contains the similar numbers and relative amounts of proteins. Thus, differences in protein concentrations among the four MFGM preparations appear to be due largely to the amount of the contaminated immunoglobulins.

Proteins were transferred to PVDF filters and incubated with RCA-I, which binds oligosaccharides terminating with the Galβ1 → 4GlcNAc group [18]. More bands were detected with lectin binding than CBB staining. However, no differences in the lectin binding patterns were observed for the four preparations except for immunoglobulin light and heavy chain bands (Fig. 1B). Digestion of MFGM glycoproteins with diplococcal β-galactosidase prior to the analysis abolished the lectin binding to all of the bands (data not shown), indicating that RCA-I is binding to oligosaccharides terminating with a β-1,4-linked galactose residue. When the filter was incubated with SBA, which binds oligosaccharides terminating with either an α- or β-*N*-acetylgalactosamine residue or, more weakly, with an α-galactose residue [19], reactivity of the lectin with 47K and 80K bands

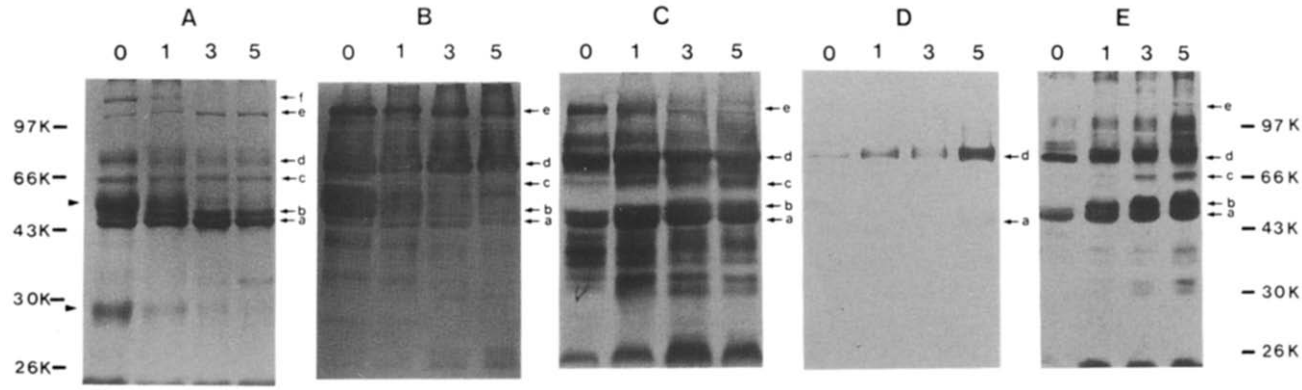

Fig. 1. Western blot analysis of MFGM glycoproteins using lectins. MFGM glycoproteins prepared from milk at day 0, day 1, day 3 and day 5 after parturition were subjected to SDS-PAGE followed by CBB staining (A) or Western blot analysis using RCA-I (B), WGA (C) or SBA (D and E). In panel E, the filter was treated with sialidase prior to incubation with SBA. The numbers at the top of the filters indicate days of milk from which MFGM glycoproteins were prepared. The arrowheads indicate the positions of immunoglobulin heavy and light chains. The a, b, c, d, and e in each panel and f in panel A indicate the positions of 47K, 50K, 66K, 80K, 120K and 160K bands, respectively.

increased during the lactation (Fig. 1D). Since CBB-staining and RCA-I binding indicated that these two bands are present in the similar relative ratios in the four preparations, it was assumed that this variation in lectin binding is induced by an increase of SBA-positive oligosaccharides and/or an uncovering of SBA-positive oligosaccharides in the bands during the lactation. Because a slight but significant decrease in the binding of WGA, which binds oligosaccharides terminating with sialic acid [20], was observed for several glycoproteins including 47K and 80K bands during the lactation (Fig. 1C), it was suggested that a decrease in sialylation is partly responsible for the increased SBA binding.

To confirm this, the filter was treated with *A. ureafaciens* sialidase, which cleaves all sialyl linkages, prior to the lectin blotting. The result showed that a lactation-dependent variation in SBA-binding is profoundly augmented not only in 47K and 80K bands but also in 30K, 33K, 38K, 50K, 66K, 87K and 100K bands after sialidase treatment (Fig. 1E). Most of the SBA-positive bands were present in all four preparations as detected with CBB staining, RCA-I binding or WGA-binding (Fig. 1A, B or C). Therefore, these results indicated that a lactation-dependent increase of SBA binding to these bands could be ascribed to the combination events of a decrease in sialylation of SBA-positive oligosaccharides and an increase of SBA-positive oligosaccharides in the protein bands. Since SBA binding disappeared after digestion of the MFGM samples with jack bean β-*N*-acetylhexosaminidase but not with *Acremonium* sp. α-*N*-acetylgalactosaminidase or coffee bean α-galactosidase (data not shown), it was concluded that SBA was binding to a terminal β-*N*-acetylgalactosamine residue.

Oligosaccharides were released by hydrazinolysis from MFGM preparations from day-1 and day-5 milk and reduced with NaB^3H_4. Similar amounts of radioactivity were incorporated into the two preparations, indicating that day-1 and day-5 MFGM glycoproteins contain similar amounts of oligosaccharides. Radioactive oligosaccharides obtained from the two preparations were subjected to paper electrophoresis, and separated into neutral (N) and acidic (A) fractions (Fig. 2a and c, respectively). The percent molar ratios of fractions N and A calculated from their radioactivities were 26% and 74% for the day-1 preparation and 41% and 59% for day-5 preparation, respectively. When fractions A from both preparations were digested exhaustively with *A. ureafaciens* sialidase, all of the acidic oligosaccharides were converted to neutral ones (AN) (Fig. 2b and d, respectively). Therefore, most of the acidic nature of the oligosaccharides could be ascribed to their sialic acid residues. These results indicated that sialylation of MFGM glycoproteins decreases during early stages of lactation, which agrees with the WGA binding patterns shown in Fig. 1C.

When oligosaccharides in fractions N and AN were

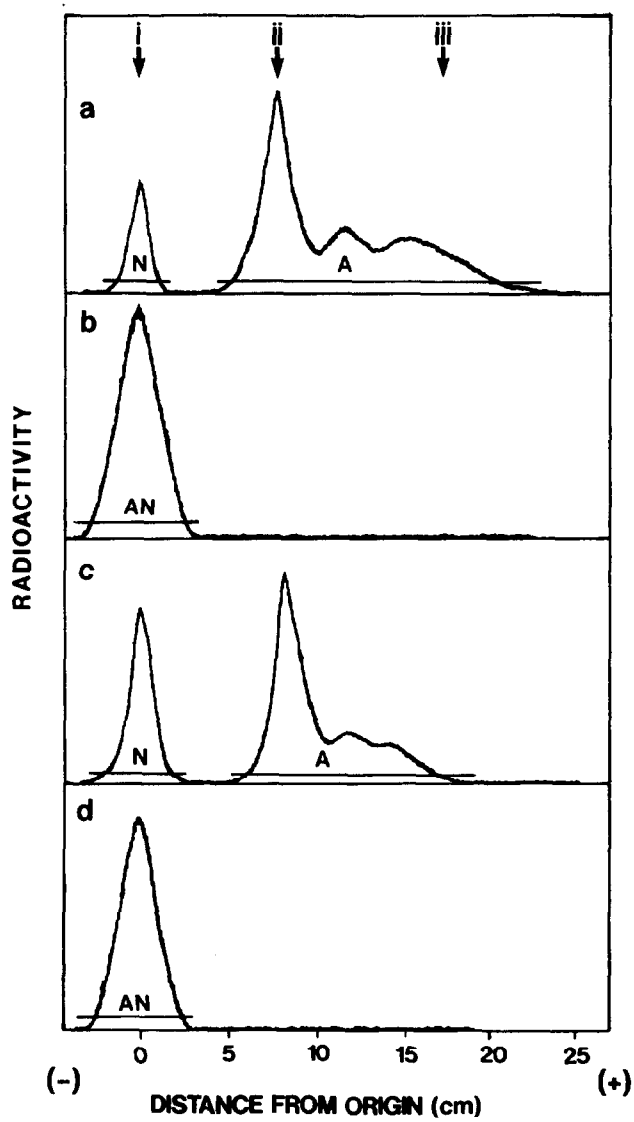

Fig. 2. Paper electrophoretograms of oligosaccharides released from MFGM glycoproteins. The radioactive oligosaccharides obtained from MFGM glycoproteins prepared from day-1 and day-5 milk samples were subjected to paper electrophoresis (a and c, respectively). Fractions N and A were recovered from the paper by elution with water. Both fractions A were digested with *A. ureafaciens* sialidase and analyzed by paper electrophoresis (b and d, respectively). The arrows at the top of the figure indicate the migrating positions of authentic oligosaccharides: (i), lactitol; (ii) and (iii), mono- and di-sialyl biantennary complex-type sugar chains obtained from human transferrin, respectively.

combined and subjected to SBA-agarose column chromatography, 14.4% and 34.6% of the total oligosaccharides from day-1 and day-5 MFGM preparations, respectively, were retarded or bound to the column and the bound oligosaccharides were eluted with 100 mM *N*-acetylgalactosamine. The result indicated that the amount of SBA-positive oligosaccharides in MFGM glycoproteins is increased during lactation, which agrees with the SBA binding patterns shown in Fig. 1E.

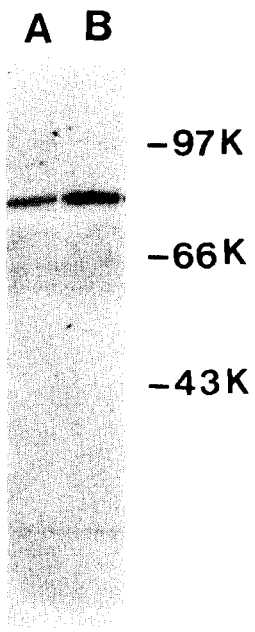

Fig. 3. Identification of a major SBA-positive band. SBA-positive glycoproteins from day-5 MFGM preparation were obtained by SBA-agarose affinity precipitation and analyzed by Western blotting using an anti-CD36 antibody (lane A). Bovine MFGM CD36 was purified by the method described previously [21] and is shown in lane B as a control.

Since the molecular weight of a major SBA-positive band, 80K (Fig. 1D) is very close to that of CD36 [21], glycoproteins precipitated with SBA-agarose beads were subjected to Western blot analysis using a specific anti-bovine CD36 antibody. The result showed antibody binding to the 80K band (Fig. 3), suggesting that the major SBA-positive band in Fig. 1D is CD36.

Recently bovine MFGM CD36 has been shown to contain *N*-linked sugar chains terminating with sialylated and non-sialylated GalNAcβ1 $\rightarrow$ 4GlcNAc and Galβ1 $\rightarrow$ 4GlcNAc groups [22]. Because SBA also binds to the GalNAcβ1 $\rightarrow$ 4-GlcNAc group and because all of the SBA-positive bands disappeared when MFGM glycoproteins were treated with jack-bean β-*N*-acetylhexosaminidase or *N*-glycanase (data not shown), it was concluded that SBA is binding to *N*-linked sugar chains of MFGM glycoproteins terminating with the GalNAcβ1 $\rightarrow$ 4GlcNAc group. Increases of SBA-positive oligosaccharides and of SBA-positive proteins during the lactation indicated that *N*-acetylgalactosaminyltransferase responsible for forming the disaccharide is activated at a very early stage of lactation.

Because RCA-I binding patterns of MFGM glycoproteins among the four preparations were not largely changed, the amounts of sialic acid residues bound to galactose residues do not appear to change significantly during the lactation. Therefore, the decrease in sialic acid residues appeared to be specific to oligosaccharides which contain SBA-positive structures. Methylation analysis showed that all of the acidic oligosaccharides of MFGM glycoproteins contain only α-2,6-linked sialic acid residue (Sato, T. and Furukawa, K., unpublished data). Therefore, the present results indicated that the amount of the Neu5Acα2 $\rightarrow$ 6GalNAcβ1 $\rightarrow$ 4GlcNAc group is decreased, while that of the Neu5Acα2 $\rightarrow$ 6Galβ1 $\rightarrow$4GlcNAc group is unchanged in MFGM glycoproteins during the lactation. The decreased sialylation of the SBA-positive oligosaccharides observed in this study could be induced by activation of a sialidase with a restricted substrate specificity and/or by lowered activity of an α-2,6-sialyltransferase. It would be of interest to determine how the sialylation levels of the GalNAcβ1 $\rightarrow$ 4GlcNAc and Galβ1 $\rightarrow$ 4GlcNAc groups of MFGM glycoprotein sugar chains are regulated during lactation.

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